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14. ABSTRACT

Epidermal growth factor (EGF) receptor tyrosine kinases (erbB family), EGFR (erbB1) and HER2, are highly expressed in breast cancer and are associated with poor prognosis. A number of EGFR and/or HER2-targeted agents are being investigated for breast cancer treatment. Brk (Breast Tumor Kinase) is a non-receptor tyrosine kinase that has been shown to enhance the mitogenic signaling of EGF, induce phosphorylation of erbB 3 and interact with AKT. In this study, we aim to investigate whether Brk can promote cells to become refractory to EGFR-targeted drugs. PI-3 kinase/AKT pathway mediates EGF-induced cell growth and survival and is involved in cellular resistance to anti-cancer drugs. Because the PI3K/AKT pathway is regulated by multiple activators, downregulation of the EGFR alone may not lead to its inhibition. We will investigate whether Brk promotes growth and survival as well as PI3K/AKT activity in cells treated with EGFR-targeted agents.

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Introduction

Epidermal growth factor (EGF) receptor tyrosine kinases (erbB family), EGFR (erbB1) and HER2, are highly expressed in breast cancer and are associated with poor prognosis. A number of EGFR and/or HER2-targeted agents are being investigated for breast cancer treatment. However, the redundancy of signaling pathways which promote cell growth and prevent apoptosis can cause cells to become insensitive to these drugs. Brk (Breast Tumor Kinase) is a nonreceptor tyrosine kinase that has been shown to enhance the mitogenic signaling of EGF, induce phosphorylation of erbB 3 and interact with AKT. In this study, we aim to investigate whether Brk can promote cells to become refractory to EGFR-targeted drugs. PI-3 kinase/AKT pathway mediates EGF-induced cell growth and survival and is involved in cellular resistance to anti-cancer drugs. Because the PI3K/AKT pathway is regulated by multiple activators, downregulation of the EGFR alone may not lead to its inhibition. We will investigate whether Brk promotes growth and survival as well as PI3K/AKT activity in cells treated with EGFR-targeted agents.

We propose to undertake four different tasks, as shown in the Statement of Work. The work that has been accomplished is described in Key Research Accomplishment. We have had some difficulty in two main tasks, first, the establishment of the method to suppress Brk expression in breast cancer cells utilizing RNA interference approach. Second, the establishment of breast cancer cell lines overexpressing Brk. The problems and the resolution are described in the Key Research Accomplishment. This problem has prevented us from carrying out the study at the desired speed. However, several strategies have been taken for trouble-shooting and we have accomplished a large part of the proposal. Furthermore, we have generated important data that lead to broader understanding of the function of Brk.

Statement of Work

Task 1. To determine the role of Brk on the efficacy of EGFR inhibitors to antagonize cell growth and survival (month 1- 7):

1.1). Human breast cancer cell lines will be selected by a RT-PCR screen for cells which overexpress both Brk and EGFR (month 1-2).

1.2) shRNA targeting to 'knock down' Brk gene expression will be designed and transfected into selected cell lines from 1.1. Cells will be tested for the levels of mRNA and protein expression of Brk. Subsequently, two Brk-knockdown clonal cell lines will be established (month 2-6).

1.3) The clonal cell lines, the Brk-knockdown and parental cells, will be treated with different EGFR inhibitors including AG1478, ZD1839 and GW572016. The duration of the treatment will be approximately 2 weeks. The rate of cell proliferation and apoptosis will be assessed during the course of the treatment (month 6-7).

Task 2. To determine whether Brk overexpression induces cells to become refractory to the EGFR inhibitors (month 1-7).

2.1). Human breast cancer cell lines will be selected by a RT-PCR screen for cells which contain high levels of endogenous EGFR and low or undetectable levels of endogenous Brk (month 1-2).

2.2). Brk will be transfected into selected cell lines from 2.1 and tested for the level of Brk protein expression. Two clonal cell lines overexpressing Brk will be established (month 2-6).

2.3). The clonal cell lines overexpressing Brk and parental cells will be treated with EGFR inhibitors AG1478, ZD1839, and GW 572016. The duration of the treatment will be approximately 2 weeks. The rate of cell proliferation and apoptosis will be assessed during the course of the treatment (month 6-7).

Task 3. To test whether Brk upregulates PI3K and AKT activity in the cells exposed to EGFR inhibitors (month 6-7).

The Brk knockdown, overexpressed, and parental cells, established in Task 1 and 2, will be treated with AG1478, ZD1839, or GW 572016. Subsequently, cells will be analyzed for the PI3K and AKT activity at various time-points (day1-14). The analysis will be achieved by a standard *in vitro* PI-3 kinase assay and measurement of the levels of phosphorylated AKT by immunoblotting.

Task 4. To determine the role of Brk on the efficacy of EGFR inhibitors to antagonize cell growth and survival in an *in vivo* setting (month 7-12).

4.1). The Brk-knockdown clonal cells, established in 1.2, and parental cells will be injected subcutaneously into female BALBc $^{nu/nu}$ mice and they will be monitored for tumor establishment. Two cell lines will be utilized. 12 mice will be utilized for each experimental condition. The experimental conditions will be as follow: a) mice injected with Brk-knockdown cells and treated with drug, b) mice injected with Brk-knockdown cells but not treated with drug, c) mice injected with parental cells and treated with drug, d) mice injected with parental cells but not treated with drug. Forty-eight mice will be utilized in the experiment. Two cell lines will be injected into the animals, therefore, total of 96 mice will be utilized in the entire experiment. (month 7-8)

4.2) Following tumor establishment, mice will be administered ZD1839 by oral gavage. After the course of drug treatment (3-4 weeks), mice will be sacrificed. Tumor size will be measured, and tumor growth rate will be assessed and compared between drug-treated and untreated cells (month 8-11).

Key Research Accomplishment

Task 1. To determine the role of Brk on the efficacy of EGFR inhibitors to antagonize cell growth and survival

1.1) Selection of human breast cancer cells.

Fifteen breast cancer cell lines were screen for cells that express high levels of both Brk and EGFR. These cell lines will be utilized for the investigation of the effect of Brk on cell response to EGFR inhibitors. The levels of Brk and EGFR expression in the cell lines were detected by western blot analysis. Three breast cancer cell lines were selected to be utilized in the study, the selection was based on their high levels of the expression of Brk and EGFR. These cell lines include T47D, SKBr3, and MDA-MB-231 (Fig.1).

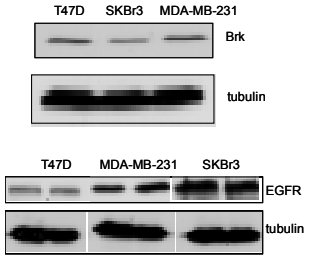

Fig. 1). Cell lines T47D, SkBr3 and MDA-MB-231 were harvested, cells were lysed and equal amount of the cell lysate were subjected to protein fractionation by SDS-PAGE. Brk, EGFR, and tubulin were detected by western blot analysis, utilizing anti-Brk, anti-EGFR and anti-tubulin antibodies.

1.2). Designing of RNA interference (RNAi) targeting to knockdown Brk

We designed 3 different shRNA sequences and constructed them into the pSUPER vector to generate pSUPER shRNA (pSUPER shRNA-Brk) targeting Brk at 3 different target sites. Additionally, a scramble RNA sequence (pSUPER shRNA-sc) were designed. The efficiency of Brk knockdown was tested by transfection of pSUPER shRNA-Brk or pSUPER shRNA-sc into the three selected breast cancer cells, T47D, SkBr3, and MDA-MB-231. Three days after the transfection, the cells were harvested and the cell lysate were subjected to analysis of Brk protein level by western blot analysis with anti-Brk antibody. All three different shRNA-Brk constructs were unable to sufficiently suppress the Brk expression (data was presented in the first annual report). We redesigned the RNA sequence by utilizing microRNA (miRNA) instead of shRNA. This method has been shown to yield better stability of the RNA interference. Three sequences of miRNA targeting to knockdown Brk and one control sequence were constructed in pSM155 vector. Subsequently, the effect of these miRNAs on Brk expression was tested. Less than 20% suppression of Brk was observed in the cells transfected with these miRNA-Brk (data not shown). Since 20% suppression of Brk is

insufficient for our purpose, we were unable to use these miRNA. To circumvent the problem, we tried the synthesized siRNA. siRNA targeting Brk (siRNA-Brk/kd) and scramble RNA (siRNA-sc) were purchased from Dharmacon Inc. The siRNA was transfected into the cell lines T47D, SkBr3 and MDA-MB 231. A significant reduction of Brk expression was observed in T47D (approximately 60% of control), and detectable level of reduction was yielded in SkBr3 and MDA-MB-231 (40-50% of control) (Fig. 2).

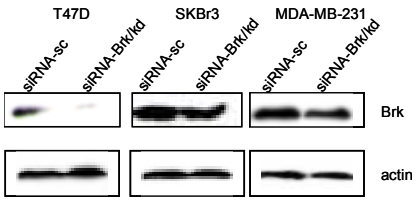

Fig. 2. Cell lines T47D, SkBr3 and MDA-MB-231 were transfected with siRNA-Brk/kd or siRNA-sc. Two days after transfection, cells were harvested, cells were lysed and equal amount of the cell lysate were subjected to protein fractionation by SDS-PAGE. Brk was detected by western blot analysis, utilizing anti-Brk, antibody. A fraction of the cell lysate from each sample was taken and loaded on a separate gel to probe for actin utilizing anti-actin antibody. This presented data represents the results of three independent experiments.

1.3) Determination of the role of Brk on cell proliferation and sensitivity of EGFR inhibitor

To determine the effect of brk knockdown on cell proliferation, we transfected the siRNA targeting Brk (siRNA-Brk/kd) or control (siRNA-sc) in three breast cancer cell lines, and assessed the number of viable cells by performing MTT assay. Brk knockdown induces significant decrease of cell proliferation in various breast cancer cell lines, as shown in Fig. 3. This result is consistent with previous report that Brk promotes cell proliferation (1). These data indicate that Brk plays an important role in the regulation of cell proliferation.

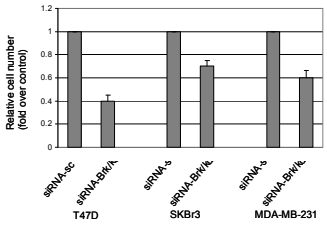

Fig. 3. Cells were transfected with siRNA-Brk or siRNA-sc. Three days after transfection cells were subjected to standard MTT assay and the MTT activity was measured by microplate

reader, to measure the relative number of viable cells. Graph represents relative number of viable cells. Data represent mean values of three independent experiments.

To investigate the role of Brk in cell sensitivity to EGFR inhibitors, were used, HER1/HER2 dual inhibitor GW2974 (Sigma-Aldrich). Since T47D is most efficiently knockdown by siRNA, and has the highest rate of cell proliferation induced by siRNABrk/kd, among the three selected cell lines, they were used for the following experiment. T47D cells were transfected with siRNA-Brk/kd or siRNA-sc and subsequently treated with ranges of concentration (20 – 80 uM) of GW2974. Afer 16 hours incubation with GW2974, cells were subjected to MTT assay. We detected a significant cell death induced by high concentration of GW2974 (60 and 80 uM) but no change was detected in cells treated with 20 and 40 uM. Knockdown of Brk did not have a significantly effect on cell death induced by GW2974 (Fig.4). If Brk has an inhibitory effect on GW2974-induced cell death, knockdown of Brk should synergize with the drug. Since the number of Brk-knockdown and control cells after drug treatment are insignificantly different, it suggests that Brk does not protect cells from GW2974-induced death. Future studies will include examination of the effect of Brk on cell sensitivity to various EGFR inhibitors AG1478, ZD1839 and GW572016.

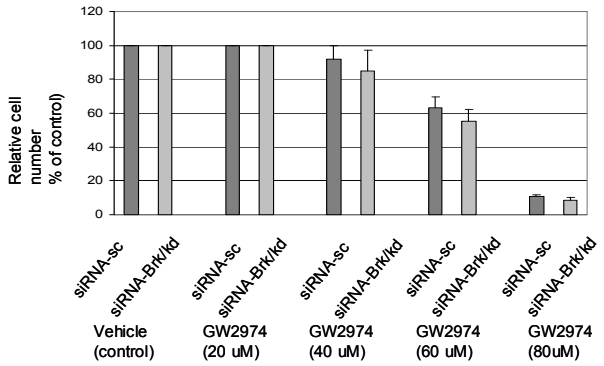

Fig. 4. T47D cells were transfected with either siRNA-Brk/kd or siRNA-sc. 36 hours following transfection, cells were incubated with GW2974, with indicated concentrations. 16 hours after the treatment, cells were subjected to MTT assay, utilizing a standard method. The absorbance was measured at 565 nm wavelength. Graph represents relative number of viable cells. Data represent mean values of three independent experiments.

Task 2 To determine whether Brk overexpression induces cells to become refractory to the EGFR inhibitors

Since knockdown of Brk induces a significant increased proliferation of T47D cells, it suggests that Brk has an important role in the regulation of cell proliferation in this cell line. We will further determine whether Brk can mediate the growth cells treated with GW2974. T47D cells were transfected with pCGNBrk or control vector. Subsequently, cells were treated with GW2974 at the concentration below IC50 (20 uM) and the treatment was maintained for 3 days. Culture media was replaced with fresh media containing GW2974 or control vehicle (DMSO) every 24 h. Following 3 days incubation, MTT assay was performed. Control cells

treated with GW2974 has a 70% reduction of number of viable cells compared to vehicle-treated cells (Fig. 5). Brk overexpressed cells that are treated with GW2974 has a 50% reduction of number of viable cells compared to Brk-overexpressed cells treated with vehicle. This decrease of cell number is not caused by cell death since we did not observe evidence of cell death. This result shows that Brk can induce cell growth in the presence of GW2974. It suggests that Brk has a mechanism of sustain cell growth that is independent of HER1/HER2. Further investigation is necessary to characterize the mechanism of Brk-induced cell growth. Furthermore, the effect of Brk overexpression on sensitivity to GW2974 of cells that express low level of endogenous Brk will be carried out in the future. We have screen breast cancer cell lines and selected three cell lines that express low level of Brk and high level of EGFR. These cell lines include BT474, MDA-MB-468 and MCF10A . Presently, we are in the process of establishing the clonal cell ines overexpressing Brk. We have some technical problems with generating these cell lines, which has delayed our proposal plans. Once successfully established, the cell lines will be utilized to examine the effect of Brk overexpression on cell sensitivity to GW2974 will be examined.

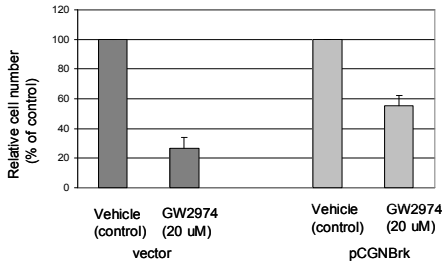

Fig. 5 pCGNBrk or pCGN vector was transfected into T47D cells. 24 hour after transfection, cells were treated with GW2974 (20 uM) or control vehicle (DMSO). Cells were maintained for 3 days, media was replaced with fresh media containing GW2974 or control vehicle (DMSO) every 24 h. Following 3 days incubation, MTT assay was performed. The absorbance was read by microplate reader at 565 nm wavelength. Graph represents relative number of viable cells. Data represent mean values of three independent experiments.

Task 3 Determination of the role of Brk in the regulation of Akt activity

Akt regulates cell growth and survival and induces cell resistance to cancer drugs. Since Brk signaling pathway is associated with Akt, we will examine whether Brk can induce Akt activation and whether the mechanism is dependent on EGFR (2,3,4). We have reported in the first annual report that Brk promotes the activation of Akt as judged by the increase of phosphorylation of the regulatory site of Akt, serine-473. Furthermore, we found that, in MDA-MB-231 cells, this increase of AKT phosphorylation appears to be EGF independent, since there was a detectable increase in cells expressing Brk compared to control in the absence of EGF stimulation. In T47D cells, however, the increase of AKT phosphorylation is EGF dependent. Since the activation of Akt in T47D cells appear to depend on EGF, we further define the pathway, as to whether it is dependent on HER1 and HER2. T47D cells were transfected with pCGNBrk or pCGN vector. One day after transfection, cells were treated with GW2974 and incubated for 16 hours. Cells were harvested and the cell lysate were subjected to

protein fractionation by SDS-PAGE and AKT was detected by immunoblot analysis using an antibody specific to AKT. The phosphorylated form of Akt-serine-473 was detected by the antibody specific to phosphor serine 473 of Akt. The result showed a detectable increase of AKT phosphorylation in cells transfected with Brk compared to control and this increase is abolished when treated with GW2974. This result suggests that the regulation of Akt by Brk is dependent on HER1 and HER2. Future study will include characterization of the mechanism of EGF-independent regulation of Akt by Brk in MDA-MB-231.

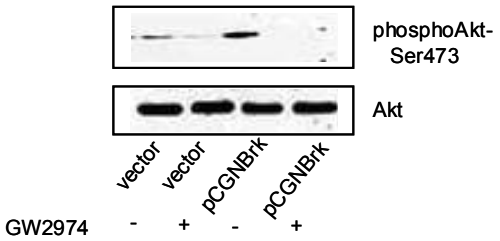

Fig. 6 T47D cells were transfected with pCGNBrk or pCGN vector. One day after transfection, cells were treated with GW2974 and incubated for 16 hours. Cells were harvested and equal amount of the cell lysate were subjected to protein fractionation by SDS-PAGE and western blot analysis. The phosphorylated form of Akt-serine-473 was detected by the antibody specific to phospho-Akt-Ser-473. A fraction of the cell lysate from each sample was taken and loaded on a separate gel to probe with anti- AKT antibody. This data represents the results of three independent experiments.

Conclusion

The finding from this current report include
1. Brk promotes cell proliferation.
2. Brk does not protect cells from GW2974-induced death.
3. Brk can induce cell growth in the presence of GW2974.
4. The regulation of Akt activity by Brk is dependent on EGF signaling, HER1 and HER2 in T47D cell, whereas it is independent of EGF signaling in MDA-MB-231 cells.

References

1. Harvey AJ and Crompton MR (2004) The Brk protein tyrosine kinase as a therapeutic target in cancer opportunities and challenges. Anticancer Drugs. 2004 Feb;15(2):107-11. Review
2. Kamalati T, Jolin HE, Mitchell PJ, Barker KT, Jackson LE, Dean CJ, PAGE MJ, Gusterson BA, Crompton MR (1996) Brk, a breast tumor-derived non-receptor protein-tyrosine kinase,

sensitizes mammary epithelial cells to epidermal growth factor. J Biol Chem. 29;271(48):30956-63.
3. Kamalati T, Jolin HE, Fry MJ & Crompton MR. (2000) Expression of the BRK tyrosine kinase in mammary epithelial cells enhances the coupling of EGF signalling to PI 3-kinase and Akt, via *erb*B3 phosphorylation Oncogene 19: 5471−5476.
4. Zhang P, Ostrander JH, Faivre EJ, Olsen A, Fitzsimmons D, Lange CA, (2005) Regulated association of protein kinase B/Akt with breast tumor kinase. J Biol Chem. 21;280(3):1982-91